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(54) Zirconium oxide and related compounds for purification of nucleic acids

(57) The present invention relates to unique compositions which bind nucleic acids. The compositions are useful in processes for purifying nucleic acid from a sample.

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Description

BACKGROUND OF THE INVENTION

[0001] The present invention relates generally to purification of nucleic acids by solid phase extraction, and more specifically to zirconium oxide and related surfaces which are capable of binding and eluting nucleic acids under suitable conditions.

[0002] The preparation and purification of high-purity double-stranded (ds) plasmid DNA, single-stranded (ss) phage DNA, chromosomal DNA, agarose gel-purified DNA fragments and RNA is of critical importance in molecular biology. Ideally, a method for purifying nucleic acids should be simple, rapid and require little, if any, additional sample manipulation. Nucleic acids rendered by such a method should be immediately amenable to transformation, restriction analysis, litigation or sequencing. A method with all of these features would be extremely attractive in the automation of nucleic acid sample preparation, a goal of research and diagnostic laboratories.

[0003] Typically, the preparation of plasmid DNA from crude alcohol precipitates is laborious, most often utilizing CsCl gradients, gel filtration, ion exchange chromatography, or RNase, proteinase K and repeated alcohol precipitation steps. These methods also require considerable downstream sample preparation to remove CsCl and other salts, ethidium bromide and alcohol. Similar arguments extend when using any of these methods for purifying DNA fragments. A further problem with these methods is that small, negatively-charged cellular components can copurify with the DNA. Thus, the DNA can have an undesirable level of contamination.

[0004] Nucleic acids can also be purified using solid phases. Conventional solid phase extraction techniques have utilized surfaces which either (1) fail to attract and hold sufficient quantities of nucleic acid molecules because of surface design to permit easy recovery of the nucleic acid molecules during elution, or (2) excessively adhere nucleic acid molecules to the surface, thereby hindering recovery of the nucleic acid molecules during elution. Conventional metal surfaces which cause these problems when utilized in solid phase extraction include silica surfaces such as glass and Celite. Adequate binding of nucleic acids to these types of surfaces can be achieved only by utilizing high concentrations of chaotropes or alcohols which are generally toxic, caustic, and/or expensive. For example, it is known that DNA will bind to crushed glass powders and to glass fiber filters in the presence of chaotropes. The chaotropic ions typically are washed away with alcohol, and the DNAs are eluted with low-salt solutions or water. Importantly, RNA and protein do not bind. However, a serious drawback in the use of crushed glass powder is that its binding capacity is low. In addition, glass powders often suffer from inconsistent recovery, incompatibility with borate buffers and a tendency to nick large DNAs. Similarly, glass fiber filters provide a nonporous surface with low DNA binding capacity. Other silicas, such as silica gel and glass beads, are not suitable for DNA binding and recovery. Currently, the solid phase of choice for solid phase extraction of DNA is Celite such as found in Prep-A-Gene™ by Bio-Rad Laboratories. As with the crushed glass powders, high concentrations of chaotropes are required for adequate binding of the DNA to the Celite.

[0005] There are numerous protocols for purifying DNA. For example, U.S. Patent 4,923,978 discloses a process for purifying DNA in which a solution of protein and DNA is passed over a hydroxylated support and the protein is bound and the DNA is eluted. U.S. Patent 4,935,342 discloses purification of DNA by selective binding of DNA to anion exchangers and subsequent elution. U.S. Patent 4,946,952 discloses DNA isolation by precipitation with water-soluble ketones. A DNA purification procedure using chaotropes and dialyzed DNA is disclosed in U.S. Patent 4,900,677.

[0006] Diatoms have also been utilized for purification of nucleic acids as evidenced by U.S. Patent No. 5,234,809 to Boom et al. and U.S. Patent No. 5,075,430 to Little et al.

SUMMARY OF THE INVENTION

[0007] In order to provide a more effective and efficient technique for the purification of nucleic acids, the present invention relates to a zirconium oxide composition which may be modified by refluxing zirconium oxide with sodium hydroxide. This zirconium oxide composition is particularly useful for purification of nucleic acids from other cellular components. Other related compositions which were also found to be useful in such nucleic acid purification methods include hafnium oxide and aluminum oxide. All of these metal oxide compositions are useful for nucleic acid purification in either hydrated or unhydrated form.

[0008] Such a nucleic acid purification process involves exposure of the zirconium oxide or related composition to a sample containing cellular components and then separating the composition from the sample. Nucleic acids in the sample are bound to the zirconium oxide or related composition, and can be recovered by elution with a suitable elution buffer.

[0009] When used in nucleic acid purification processes, the zirconium oxide or related composition of the present invention may be in the form of particles or beads for ease of packaging in a kit format.

DETAILED DESCRIPTION OF THE INVENTION

[0010] The present invention relates to unique compositions of matter. The compositions of matter include a zirconium oxide composition, a hafnium oxide composition and an aluminum oxide composition, all of which bind nucleic acids. The nucleic acid bound to these compositions may be eluted therefrom.

[0011] The compositions of the present invention may be modified by refluxing a starting material, such as zirconium oxide, hafnium oxide or aluminum oxide with a suitable alkaline substance such as sodium hydroxide. Suitable starting materials for use in the refluxing reaction are commercially available from numerous sources in a particulate or pelleted form. However, any form or shape of starting material is suitable for refluxation to produce a hydrated form of the composition of the present invention.

[0012] The refluxing of the starting material is with a suitable alkaline substance. Such a substance is a composition that contributes to the resultant hydrated composition of the present invention being sufficiently electropositive to bind nucleic acids which are relatively electronegative. Most strong bases in water will be suitable alkaline substances for refluxing with starting material to yield the hydrated composition of the present invention. Examples of such suitable alkaline substances include sodium hydroxide, potassium hydroxide or any other base which generates a hydroxide ion.

[0013] Subsequent reactions to which the bound nucleic acid may be subjected may determine which alkaline substance is preferred. For example, because sodium ions are believed to inhibit Strand Displacement Amplification ("SDA") reactions, a hydroxide ion donor other than sodium hydroxide, such as potassium hydroxide, may be preferred if the nucleic acids bound to the hydrated zirconium silicate composition are to be amplified by SDA.

[0014] The amount of alkaline substance used in the refluxing reaction is generally about 0.1M equivalents to about 5M equivalents of the amount of starting material used in the reaction.

[0015] The refluxing reaction used to produce the hydrated compositions of the present invention is generally conducted for about 18 to about 96 hours at about 100°C (*i.e.* reflux). Following such refluxation, the resultant hydrated composition may optionally be filtered and washed with aqueous acid such as sulfuric acid or hydrochloric acid. An alternative option is to use such aqueous acids to acidity the refluxed suspension of hydrated composition to a pH of 6.0 or lower, and then filter and wash the hydrated composition with a suitable wash buffer such as water and/or acetone.

[0016] The hydrated composition produced by the above-described reflux reaction binds nucleic acid. It is believed that the binding of nucleic acid is due at least in part to the attraction of the electropositive hydrated composition for electronegative nucleic acids. More specifically, the positively charged atoms, for instance Hf^{4+} from HfO_2, Zr^{4+} from ZrO_2 and Al^{3+} from Al_2O_3 provide sufficient electropositivity to the hydrated composition to interact with the negatively charged-phosphates of the nucleic acids, thus causing binding.

[0017] As set forth in greater detail in the Examples below, the ability of the hydrated compositions as well as the unhydrated compositions of the present invention to bind nucleic acids is quantifiable by the percentage of nucleic acid bound to the surface from an otherwise clean simple (*i.e.* without the non-target nucleic acids) under certain conditions. Due to the generally small volumes of samples from which nucleic acids are purified, the percentage of bound nucleic acid is based on a small amount of hydrated or unhydrated composition (*i.e.* 2.5mg), and a total amount of nucleic acid which is generally found in a typical sample from which nucleic acid is purified (*i.e.* about 10^4 target nucleic acid molecules).

[0018] Thus, in order to have some utility in a process of purifying nucleic acid from a sample, a nucleic acid binding composition present in an amount of about 2.5mg in a sample containing about 10^4 target nucleic acid copies should bind at least about 85 percent of the target nucleic acid copies. As shown in the Examples below, the hydrated and unhydrated composition of the present invention meet this requirement.

[0019] The nucleic acids which are bound by the compositions of the present invention include DNA and RNA obtained from any source, including but not limited to crude cell extracts, biological fluids, phage supernatants, agarose gels and radiolabelling reactions. The nucleic acid, particularly DNA can be double-stranded, single-stranded, circular or linear, and can be variable in size. Conventional techniques for obtaining nucleic acid from any source, well known in the art, are utilized to prepare the nucleic acid for purification. Typical procedures for obtaining nucleic acid end with a suspension of the nucleic acid in solution. For isolation of nucleic acid from biological samples, see, e.g., Harding, J.D. et al., Nucleic Acids Research 17:6947 (1989) and Marko, M.A. et al., Analytical Biochemistry 121:382 (1982). Procedures for isolation of plasmid DNA can be found in Lutze, L.H. et al., Nucleic Acids Research 20:6150 (1990). Techniques for extraction of double-stranded DNA from biological samples can be found in Yamada, O. et al., Journal of Virological Methods 27:203 (1990). Most DNA solutions comprise the DNA in a suitable buffer such as TE (Tris-EDTA), TEA (409 mm Tris-acetate, 1 mm EDTA) buffer, or a lysate. See also Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, New York (1989).

[0020] Once the nucleic acid is obtained in a suitable sample form, generally suspension or solution, a composition of the present invention is exposed to the sample for a period of time sufficient for nucleic acid in the sample to bind to the composition. Generally, such exposure is for a time period of about 30 seconds to about 60 minutes at a tempera-

ture of about 0°C to about 25°C. A suitable binding buffer, such as, for example, water and acidic buffers with pHs below about 7.0, may be used to assist the binding of nucleic acid to the composition.

[0021] The composition with nucleic acid bound thereto is then separated from the sample. Separation is generally accomplished by centrifugation or filtration.

[0022] The composition may then be washed, and the nucleic acid may be recovered from the composition. Washing is generally performed with low molecular weight alcohols or other suitable washing solutions such as 80%/20% ethanol/50mM Tris, pH 7.0, water or any low pH buffer.

[0023] The recovery of bound nucleic acid from composition is generally conducted by elution with a suitable elution buffer. Suitable elution buffers include most basic buffers with pHs above about 7.0 such as, for example, any low salt buffer such as 50mM Tris, KPI and KPDG. Preferably, the elution buffer will have minimal interference with processes to which the recovered nucleic acid may be subjected and have minimal negative environmental effects. Typically, elution recovery of nucleic acids from composition is conducted over a time period of about one minute to about 60 minutes at a temperature from about 4°C to about 95°C. One way in which such elution may be conducted is to flow the elution buffer past the composition with nucleic acid bound thereto to cause the elution of nucleic acid. Alternatively, the composition with nucleic acid bound thereto may be placed into the elution buffer, and then separated from the elution buffer in order that the nucleic acid may be recovered from the elution buffer.

[0024] The nucleic acid obtained by purification with a composition of the present invention may be used without further manipulation for restriction enzyme digestion, cloning, sequencing, diagnostics and the like. The high quality of nucleic acid prepared with the present invention and the speed with which nucleic acid is purified with minimal downstream processing mean that the composition can be useful in the automation of nucleic acid sample preparation.

[0025] One particularly useful form for the compositions of the present invention for automated nucleic acid sample preparation is as particles. Particulation of the composition provides maximal surface area for binding of nucleic acid. The particles may be of various shapes, including for example, spheres, cubes, oval, capsule-shaped, tablet-shaped, non-descript random shaped, etc., and may be of uniform shape or non-uniform shapes. Whatever the shape of a particle, its diameter at its widest point is generally in the range of from about 0.1 mm to about 0.15 mm. Pellets or beads of a generally spherical shape are the preferred particulate form of the composition of the present invention.

[0026] The hydrated forms of the compositions of the present invention are also particularly useful in a particle form because of their density range of about 1.0mg/uL to about 4.0mg/uL with a preferred value of about 3.3mg/uL. For comparison, hydrated Celite has a density of about 0.4mg/uL. This unusually high density of the hydrated forms of the compositions of the present invention renders it easier to separate the particles of such compositions with bound nucleic acid from the sample by centrifugation. Also, due to the density of the particles, the pellet formed by the centrifugation is tighter than with less dense compounds allowing a longer time, if desired, between centrifugation and supernatant (sample) removal, as well as an ability to remove more of the supernatant (sample) without dislodging portions of the pellet. The ability to remove more of the supernatant is particularly important as more of the cellular debris and binding buffer are removed, both of which may inhibit or interfere with subsequent processing of the purified nucleic acids.

[0027] Particulate forms of the compositions of the present invention are also advantageous due to their ease of use in kits. Particles can be easily and efficiently packaged in any suitable container as a kit or part of a kit for purification of nucleic acids from samples. Suitable containers for packaging particles of the compositions of the present invention include vials, tubes, evacuated tubes or containers, and other containers to which a sample containing nucleic acids may be added. Such kits may also include other containers of binding buffer, wash buffer and/or elution buffer and any other components desired as part of a process to purify nucleic acids from a sample.

[0028] The following examples illustrate specific embodiments of the invention described in this document. As would be apparent to skilled artisans, various changes and modifications are possible and are contemplated within the scope of the invention described.

EXAMPLE 1

Production of Hydrated Zirconium Oxide Composition

[0029] A four gram aliquot of zirconium oxide beads from Aldrich was added to 80mL of water containing 5.2 grams sodium hydroxide (NaOH). The resulting suspension was refluxed for 68 hours, and then cooled to room temperature and centrifuged at 1800 rpm for 3.5 minutes, and the supernatant removed and discarded. The suspension was then rendered acidic (pH of about 2.0) by addition of 20ml of 1% sulfuric acid (H_2SO_4) and 5mL of 10% H_2SO_4. Alternatively, the solid is isolated from solution, and then added to the acidic solution. This acidic suspension was rocked at room temperature for one hour, and then centrifuged at 1800 rpm for 3.5 minutes, and the supernatant removed and discarded. To each tube, 20ml of water was added, and the suspensions vortexed and filtered followed by washes with 100ml of water and 100ml of acetone. Finally, the powders were air dried for one hour and then oven dried for 16 hours at 100°C.

[0030] The resultant hydrated zirconium oxide powders were sufficiently electropositive to bind nucleic acids which could then be easily eluted therefrom as shown in the subsequent Examples.

EXAMPLE 2

Production of Hydrated Hafnium Oxide Composition

[0031] A four gram aliquot of hafnium oxide beads from Aldrich was added to 80ml of water containing 3.04 grams sodium hydroxide (NaOH). Then, the same procedure set forth in Example 1 was followed to yield hydrated hafnium oxide beads sufficiently electropositive to bind nucleic acids which could then be easily eluted therefrom as shown in subsequent Examples.

EXAMPLE 3

Production of Hydrated Aluminum Oxide Composition

[0032] A four gram aliquot of aluminum oxide beads from Aldrich was added to 80ml of water containing 6.28 grams sodium hydroxide (NaOH). Then, the same procedure set forth in Example 1 was followed to yield hydrated aluminum oxide beads sufficiently electropositive to bind nucleic acids which could then be easily eluted therefrom as shown in subsequent Examples.

EXAMPLE 4

Binding and Elution of Nucleic Acid To and From Hydrated Compositions

[0033] This experiment was performed to demonstrate the nucleic acid binding and elution characteristics of hydrated and unhydrated zirconium oxide, hafnium oxide and aluminum oxide.

[0034] The compositions of Example 1, 2 and 3 (hydrated zirconium oxide, hydrated hafnium oxide and hydrated aluminum oxide, respectively) as well as unhydrated forms of these three compositions were mixed with 200µL water followed by the addition of 1µL of 10^6 P^{32} labeled *M. tuberculosis* DNA. The resulting suspensions were incubated at room temperature 30 minutes on a rotator device. Following centrifugation, the supernatents were removed and added to 10mL of scintillation fluid for counting. 200µL of water was added to the remaining pellets and the resulting mixtures were incubated at 65°C for 30 minutes. Following centrifugation the supernatents were counted and the elution step repeated.

[0035] The results are shown below.

Conclusions

[0036] Although the unhydrated zirconium oxide, hafnium oxide and aluminum oxide compositions are useful for nucleic acid purification, their hydrated counterparts are better for such purpose because of greater binding and elution of nucleic acid under comparable conditions. Also, the hydrated counterparts showed comparable nucleic acid purification properties to hydrated zirconium silicate compositions.

EXAMPLE 5

Increasing pH Effect or Elution of Nucleic Acid from Hydrated Compositions

[0037] This experiment was performed to determine if nucleic bound to hydrated zirconium silicate beads (comparative composition of Example 4) would elute at various pHs.

[0038] The same protocol as used in Example 4 was used to bind *M. tuberculosis* DNA to 10mg of beads of hydrated zirconium silicate, except that following the first centrifugation, in addition to water, different concentrations of NaOH were used as elution buffers. The results are set forth below.

REACTION No.	ELUTION BUFFER	CPM SUPER BINDING	CPM SUPER ELUTION #1	CPM SUPER ELUTION #2	% DNA BOUND	% DNA ELUTED
1	Water	461	542	234	100	1
2	NaOH pH10	422	55140	9767	100	93
3	NaOH pH12	369	70322	3096	100	100
4	NaOH 1N	303	71518	3755	100	100
CONTROL NO SURFACE ADDED						
5		69619				

Conclusions

[0039] There does seem to be a significant correlation between pH of the elution buffer and percentage DNA eluted, although other factors may be involved. Also, due to the similar nucleic acid purification properties of the other hydrated compositions as shown in Example 4, it is expected that such hydrated compositions would show similar correlation between pH of the elution buffer and percentage DNA eluted.

EXAMPLE 6

Effects of Elution Buffers on Nucleic Acid Bound to Hydrated Compositions

[0040] This experiment was performed to determine if nucleic acid bound to hydrated zirconium silicate beads would elute in various buffers.

[0041] 200 uL water was added to a centrifuge tube containing 10mg of hydrated zirconium silicate beads followed by 1uL of DNA (10^4 copies of *M. tuberculosis* genomic DNA). The resulting suspension was incubated at room temperature on a rotator device for 30 minutes. Following centrifugation, the supernate was removed and added to 10mL scintillation fluid and counted. 200uL of buffer was added to the pellet. The resulting suspension was incubated at 65°C for 30 minutes. Following centriffugation the supernate was removed and counted as above and the elution step repeated. The results are shown below.

REACTION NO.	ELUTION BUFFER	CPM SUPER BINDING	CPM SUPER ELUTION #1	CPM SUPER ELUTION #2	% DNA BOUND	% DNA ELUTED
1	Water	565	668	266	100	1
2	NaOH pH7.2	573	4053	5254	100	11
3	NaHCO3 pH9.4	579	61630	3996	100	81
4	25MMKPI pH7.6	414	63961	5055	100	85
5	KPDG pH7.6	475	64701	2564	100	83
CONTROL NO SURFACE ADDED						
6		81466				

Conclusions

[0042] The buffers used elute much more DNA than does water. As in Example 5, due to the similar nucleic acid purification properties of the other hydrated compositions as shown in Example 4, it is expected that such hydrated compo-

sitions would show similar elution characteristics to the hydrated zirconium silicate.

EXAMPLE 7

Nucleic Acid Binding Conditions for Hydrated Compositions

[0043] This experiment was performed to determine preferred binding conditions for nucleic acid to 5mg of hydrated zirconium silicate composition.

[0044] The same protocol as used in Example 4 was followed except that binding buffers and incubation times were used as shown below, and elution centrifugations were not performed. The results are shown below.

REACTION NO.	BINDING BUFFER	BINDING TEMP C	CPM SUPER BINDING	% DNA BOUND
1	H20	4	630	97
2	H20	25	506	98
3	H20	37	1098	96
4	25MM KPI pH 7.6	4	21352	0
5	25MM KPI pH 7.6	25	22045	0
6	25MM KPI pH 7.6	37	20302	0
7	HCl pH 4	4	70	100
8	HCl pH 4	25	543	98
9	HCl pH 4,	37	401	98
10	NaOH pH 10	4	22941	0
11	NaOH pH 10	25	19875	1
12	NaOH pH 10	37	20681	0
CONTROL NO SURFACE ADDED				
13			20802	

Conclusions

[0045] Water and HCl used in this experiment are both acidic and gave better binding of nucleic acid to hydrated zirconium silicate composition as compared to the other binding buffers, both of which are basic. Due to the similar nucleic acid purification properties of the other hydrated compositions as shown in Example 4, it is expected that acidic conditions would favorable effect binding of nucleic acid to such other hydrated compositions

[0046] Temperature, at least under conditions of this experiment, did not seem to affect binding.

EXAMPLE 8

Decreasing pH Effect on Elution of Nucleic Acid from Hydrated Compositions

[0047] This experiment was performed to determine if lowering the pH of the elution buffer will effect the efficiency of DNA elution for hydrated zirconium silicate compositions. Previously, it was shown that raising the pH had a positive effect on DNA elution from these surfaces (Example 5).

[0048] Two acids were tested at two different pH values each. Specifically, 5mg of each surface was added to 200uL of water followed by 1uL of ^{32}P labeled DNA (10^4 genomic copies of *M. tuberculosis* DNA). The resulting suspension was incubated on a rotator device for 30 minutes. Following centrifugation, the supernate was removed and added to 10mL scintillation fluid for counting. 200uL of the elution buffer was added to the remaining pellet and the resulting suspension was incubated at 65°C for 30 minutes. Following centrifugation the supernate was removed and counted as above. The results are shown below.

REACTION NO.	SURFACE	ELUTION BUFFER	CPM SUPER BINDING	% DNA BOUND	CPM SUPER ELUTION
1	Hyd $ZrSiO_4$ 080596b	H20	1581	90	730
2		HCl pH 4	134	100	1173
3		HCl pH 2	371	98	184
4		H3PO4 pH 4	319	98	830
5		H3PO4 pH 2	439	97	1002
6	Hyd $ZrSiO_4$ (7-17-96) (SFSF)b	H20	515	96	1462
7		HCl pH 4	626	95	697
8		HCl pH 2	351	98	936
9		H3PO4 pH 4	295	98	1353
10		H3PO4 pH 2	515	96	788
	Hyd $ZrSiO_4$				
11	7-8-96545a	H20	2489	86	2154
12		HCl pH 4	2322	85	1189
13		HCl pH 2	2547	87	1139
14		H3PO4 pH 4	2961	83	1784
15		H3PO4 pH 2	2912	83	1183
CONTROL NO SURFACE ADDED					
16			16906		

Conclusions

[0049] Lowering the pH appears not to affect the amount of *M. tuberculosis* eluted compared to water. It may, however, be useful for getting DNA to bind to the composition. Also, binding of DNA to the compositions is very reproducible. Similar results would be expected with other hydrated compositions based on the similar nucleic acid purification properties shown in Example 4.

[0050] While the invention has been described with some specificity, modifications apparent to those with ordinary skill in the art may be made without departing from the scope of the invention. Various features of the invention are set forth in the following claims.

Claims

1. A composition which binds nucleic acid, said composition selected from the group consisting of hydrated zirconium oxide, hydrated hafnium oxide, hydrated aluminum oxide, zirconium oxide, hafnium oxide and aluminum oxide.

2. The composition of claim 1 which binds nucleic acid in water.

3. The composition of claim 1 having a density of at least 1.0mg/uL.

4. The composition of claim 1 which in an amount of 2.5 milligrams will bind at least about 85 percent of 104 copies of a target nucleic acid in a sample without other nucleic acids.

5. A process for purifying nucleic acid from a sample comprising the steps of:

(a) exposing the sample to the composition of claim 1 for a period of time sufficient for nucleic acid in the sample to bind to said composition; and

(b) separating said composition from the sample.

6. The process of claim 6 wherein the separation of composition from sample is conducted by centrifugation or filtration.

7. The process of claim 6 further comprising:

(c) recovering nucleic acid from said composition.

8. A kit for purifying nucleic acid from a sample comprising the composition of claim 1.

9. The kit of claim 8 wherein said composition is present as particles.

10. A process for binding and subsequently eluting nucleic acid from a surface capable of binding said nucleic acid comprising the steps of:

(a) contacting said nucleic acid with said surface in the presence of a buffer solution with a pH below about 7.0; and

(b) eluting said nucleic acid from said surface in the presence of a buffer solution with a pH above about 7.0.

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PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application Number EP 98 11 5595

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X	WO 92 18514 A (MINNESOTA MINING & MFG) 29 October 1992 (1992-10-29) * page 7, line 29 - page 8, line 29 * * page 9, line 12 - page 10, line 11 * * claims 1-3; figures 3,4; examples 1,2 *	1-10	C12N15/10 C07H1/08 C12P19/34 C12Q1/68 B01J20/06
X	US 5 002 749 A (RECASENS JOSEPH ET AL) 26 March 1991 (1991-03-26) * the whole document *	1-4,8,9	
A	DE 43 09 248 A (UNGER KLAUS PROF DR ;LORENZ BERND (DE); MARME STEFAN (DE); MUELLER) 29 September 1994 (1994-09-29) * the whole document *	1-10	
T	EP 0 832 897 A (BECTON DICKINSON CO) 1 April 1998 (1998-04-01) * the whole document *	1-10	

TECHNICAL FIELDS SEARCHED (Int.Cl.6)

C12N
C07H
C12P
C12Q
B01J

INCOMPLETE SEARCH

The Search Division considers that the present application, or one or more of its claims, does/do not comply with the EPC to such an extent that a meaningful search into the state of the art cannot be carried out, or can only be carried out partially, for these claims.

Claims searched completely :

Claims searched incompletely :

Claims not searched :

Reason for the limitation of the search:

see sheet C

Place of search	Date of completion of the search	Examiner
BERLIN	27 August 2001	ALCONADA RODRIG.., A

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
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T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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INCOMPLETE SEARCH
SHEET C

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Claim(s) searched incompletely:
10

Reason for the limitation of the search:

Present claim 10 relates to a process of purifying a nucleic acid on a surface defined by reference to a desirable characteristic or property, namely, that said surface is able to bind the nucleic acid at a pH below 7.0 and is able to release the nucleic acid at a pH above 7.0. The claims cover all processes having this characteristic or property, whereas the application provides support within the meaning of Article 84 EPC and/or disclosure within the meaning of Article 83 EPC for only a very limited number of such processes. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 84 EPC). An attempt is made to define the process by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the processes of purifiying nucleic acids using a composition selected from the group of hydrated zirconium oxide, hydrated hafnium oxide, hydrated aluminim oxide, zirconium oxide, hafnium oxide and aluminium oxide (see claim 1 and example 4).

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 98 11 5595

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

27-08-2001

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9218514 A	29-10-1992	AU 1978692 A	17-11-1992
US 5002749 A	26-03-1991	FR 2629072 A	29-09-1989
		AU 3140789 A	28-09-1989
		BR 8901272 A	07-11-1989
		CA 1325879 A	11-01-1994
		CN 1036548 A,B	25-10-1989
		DE 68901991 D	13-08-1992
		DE 68901991 T	24-12-1992
		EP 0334713 A	27-09-1989
		ES 2034663 T	01-04-1993
		JP 1278419 A	08-11-1989
		JP 1777856 C	28-07-1993
		JP 4065017 B	16-10-1992
		ZA 8902122 A	29-11-1989
DE 4309248 A	29-09-1994	NONE	
EP 0832897 A	01-04-1998	CA 2214495 A	25-03-1998
		JP 10147592 A	02-06-1998

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

PREPARATION OF DNA SPECIMEN

Patent number: JP2268682
Publication date: 1990-11-02
Inventor: FUJITA MASAHIKO; others: 02
Applicant: HITACHI LTD
Classification:
- international: C12N15/10
- european:
Application number: JP19890086806 19890407
Priority number(s):

Abstract of **JP2268682**

PURPOSE:To reduce an amount of DNA adsorption and to efficiently reacting a very small amount of DNA by pouring a substance to be adsorbed on a wall of container without damaging a reaction system.

CONSTITUTION:A substance (DNA or polyoxyethylene sorbitan monolaurate, nonionic surfactant) to be adsorbed on a wall of container without damaging a reaction system is poured, a substance except DNA of specimen is adsorbed on the wall of container, an amount of DNA of specimen adsorbed on the wall of container is reduced so that reaction ratio can be improved.

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⑲日本国特許庁(JP)　⑪特許出願公開

⑫公開特許公報(A)　平2−268682

⑤1Int.Cl.⁵	識別記号	庁内整理番号	⑬公開　平成2年(1990)11月2日
C 12 N 15/10			
// C 07 H 21/04	Z	7822−4C	
		8717−4B　C 12 N 15/00	A

審査請求　未請求　請求項の数 5　(全6頁)

⑤4発明の名称　DNA試料調製方法

⑳特　願　平1−86806
㉒出　願　平1(1989)4月7日

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⑦4代理人　弁理士　小川　勝男　外1名

明　細　書

1．発明の名称

DNA試料調製方法

2．特許請求の範囲

1．容器内でDNA試料水溶液と試薬を混合して反応させるDNA試料調製方法において、反応系を阻害せず容器壁に吸着する物質を注入して反応させることを特徴とするDNA試料調製方法。

2．反応系を阻害しないDNAを注入することを特徴とする請求項1記載のDNA試料調製方法。

3．非イオン性界面活性剤を注入することを特徴とする請求項1記載のDNA試料調製方法。

4．ポリオキシエチレンソルビタンモノラウレートを用いることを特徴とする請求項3記載のDNA試料調製方法。

5．請求項1記載のDNA試料調製方法において、反応系を阻害せず容器壁に吸着する物質を注入する替わりに、反応に用いる前に容器に吸着する物質を注入して容器壁に該物質を吸着させておくことを特徴とするDNA試料調製方法。

3．発明の詳細な説明

〔産業上の利用分野〕

本発明はDNA試料調製方法に係り、特に微量DNAを自動装置において効率良く反応させるのに好適な方法に関する。

〔従来の技術〕

従来の方法は、塩基配列決定プロセスを例にとると、宝酒造株式会社のM13シークエンスキット説明書(1986年)に記載されているようにピコモル(pmole)オーダの鋳型DNA，等モル量のプライマー，バッファ，蒸留水を混合して60℃程度に加温してプライマーアニール反応を進め、前記溶液にDNAポリメラーゼとdNTP・ddNTPの混合液を加えて相補鎖合成反応を行つてきた。すなわちDNA試料の吸着防止のための工夫は特になされてなかつた。

〔発明が解決しようとする課題〕

従来法においてはDNA量が少なくなつたときに反応容器にDNAが吸着され反応率が低下する

という問題があつた。反応容器としては、安価軽量で取扱い易く、またＤＮＡ量が充分あるときはその吸着量は無視できる程度であるという理由からポリプロピレン等のプラスチック製のものが用いられている。しかしＤＮＡの絶対量が少なくなるとプラスチック壁へのＤＮＡ吸着の影響が現れて反応率が低下する。塩基配列決定プロセスにおける前記反応率の検討結果を次に示す。

第３図は標準的な条件である鋳型ＤＮＡ０．５pmole(図ｂ）とその半分の０．２５pmole（図ａ）についてのＤＮＡ断片スペクトルの比較を示したもので、各図の横軸は電気泳動時間、縦軸は蛍光強度の相対値、図中の数字はＤＮＡ断片の塩基長を示す。鋳型ＤＮＡ量を半分にするとＤＮＡ断片を示すピークのＳ／Ｎは平均して０．５pmoleの鋳型を供給したときの２０％程度にまで低下して、反応ピークの同定が困難になつた。

第４図は前記の傾向を反応率を指標にしてまとめたもので、図の横軸は鋳型ＤＮＡ供給量、縦軸は反応率の比（ここでは相対反応率と呼ぶ）で、鋳型ＤＮＡ０．４pmoleの反応率を１とした。0.4 pmole よりも鋳型ＤＮＡ量が多くなると反応率は緩やかに上昇するが、０．４pmoleよりも鋳型DNA量が少なくなるとＤＮＡの吸着現象の影響が強く現れて反応率は急激に低下する。ここでは塩基配列決定プロセスを例に述べたが、ＤＮＡ吸着は微量ＤＮＡを反応する場合すべてについて問題になる。なおＤＮＡ試料調製プロセスは操作者にとり、負担が重く、自動化の要望が強く、今後自動装置化が進展すると予想される。したがつて吸着防止法においても、自動化し易い方法を採用する必要がある。

本発明はＤＮＡ吸着量を低減して微量ＤＮＡを効率良く反応させるのに最適で、かつ自動装置において容易に実現できる方法を提供することにある。

〔課題を解決するための手段〕

本発明においては、上記目的を達成するために、反応系を阻害せず容器壁に吸着する物質を注入する方法を採用した。注入する物質としては反応系を阻害しないＤＮＡ、あるいは非イオン性界面活性剤であるポリオキシエチレンソルビタンモノラウレートを採用した。

また、上記目的を達成するために、容器壁に吸着する物質を反応前の容器に予め注入して、容器壁に前記物質を吸着させる方法を採用した。

〔作用〕

容器壁に吸着しうる分子数には上限があり、前記吸着物質の分子数をＤＮＡの分子数に対して充分大きくすれば、容器壁には試料とするＤＮＡ以外の物質が吸着することになり、試料とするDNAの容器壁への吸着量を低減でき、反応率を向上させることができる。本発明の方法は吸着物質を注入する操作が加わるだけであるので、自動化も容易である。

また反応前の容器に予め容器壁に吸着する物質を注入して、充分吸着した後に前記溶液を廃棄して、反応を行う方法については、反応前の容器壁にＤＮＡが吸着する余地が残されていないので同様に反応率を向上でき、自動化は容易である。なお本方法によると、前記溶液を充分除去してコンタミネーションの可能性を低くしておけば、容器壁に吸着する物質には反応系を阻害する性質があつても問題にならないという利点がある。

〔実施例〕

本発明によるＤＮＡ試料調製方法の塩基配列決定プロセスにおける第一の実施例を第１図のフローチャートを用いて説明する。本実施例の方法は、鋳型ＤＮＡ，鋳型ＤＮＡと等しい分子数のプライマー，バッファ，反応液量を調整する蒸留水を注入混合する分注（操作１），鋳型ＤＮＡと異なる配列で反応を阻害せず、容器壁への吸着剤として用いるＤＮＡを注入する他種ＤＮＡ注入(操作２)、５５～６０℃での温度範囲でのインキュベーション（操作３）、容器を室温レベルにまで冷却する（操作４）、ＤＮＡポリメラーゼの注入(操作５)、反応液の等分（操作６）、ＤＮＡ鎖の伸長・停止剤の注入（操作７）、室温レベルから３７℃の温度範囲でのインキュベーション（操作８）、チエイス混液の注入（操作９）、操作８と同様のイン

キュベーション（操作１０）、ホルムアミド等の停止剤の注入（操作１１）よりなる。

本方法による実験結果を第５図に示す。

本実施例ではプライマーを蛍光標識し、励起光を用いてＤＮＡ断片を発光させ、その発光を検出した。

図（ｂ）は鋳型ＤＮＡ供給量を０．２５psoleと微量にし、他種ＤＮＡを注入しないときのＤＮＡ断片スペクトルであり、図（ａ）は鋳型以外のＤＮＡとしてプライマーの分子数を鋳型ＤＮＡの分子数より一桁多く注入したときのＤＮＡ断片スペクトルである。図の横軸は泳動時間、縦軸は蛍光強度、数字はＤＮＡ断片の塩基長である。過剰に注入されたＤＮＡにより容器壁への鋳型ＤＮＡの吸着量が減り、反応率が向上していることが明白である。

本発明によるＤＮＡ試料調製方法の第二の実施例を第２図のフローチャートに示す。基本フローは第一実施例と同様で、他種ＤＮＡを注入する操作２に換えて容器壁への吸着剤として非イオン界面活性剤であるポリオキシエチレン（２０）ソルビタンモノラウレートを注入する操作１２を採用したものである。

本方法による実験結果を第６図に示す。同図（ｂ）はポリオキシエチレン（２０）ソルビタンモノラウレートを注入せずに反応を行つた場合のＤＮＡ断片スペクトル、図の上側はポリオキシエチレン（２０）ソルビタンモノラウレートをアニール反応液中で０．１％ になるよう注入したときのＤＮＡ断片スペクトルである。なおここでは、プライマーと鋳型ＤＮＡは等モル供給した。ポリオキシエチレン（２０）ソルビタンモノラウレートにより容器壁への鋳型ＤＮＡの吸着量が減り、反応率は明らかに向上した。

第一実施例，第二実施例では反応液を調製した後に吸着物質を混入したが、予め蒸留水やバッファ等に該吸着物を混合しておいても同様であることは勿論である。

本発明によるＤＮＡ試料調製方法の第三の実施例を第７図のフローチャートに示す。反応液を容器に分注・調合する操作１の前に、容器壁に吸着させ物質を容器に注入する操作１３、前記溶液を廃棄して乾燥させる操作１４を行うものである。吸着物質としてｐＢＲ３２２二本鎖ＤＮＡ並びにポリオキシエチレン（２０）ソルビタンモノラウレートを用いたところ、

第５図，第６図の上側と同等のＳ／ＮのＤＮＡ断片スペクトルが得られた。

なお以上の方法を他のＤＮＡ試料の反応，例えば制限酵素分解反応等に適用できるのは勿論である。

〔発明の効果〕

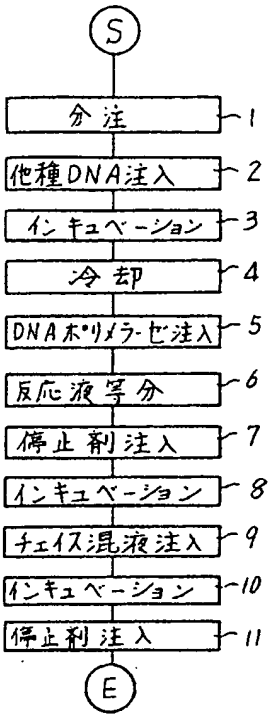
本発明では、ＤＮＡ試料の容器壁への吸着を防ぐことができ、特にＤＮＡ試料量が微量になつた場合の反応率を向上させることができる。

４．図面の簡単な説明

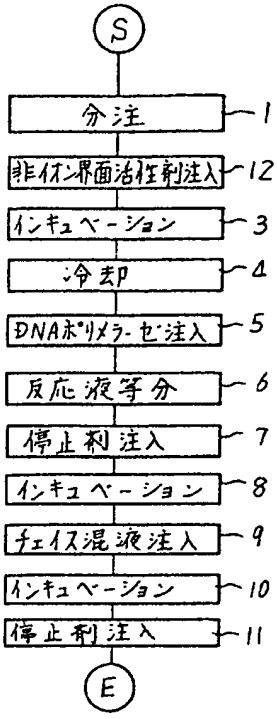
第１図，第２図，第７図は本発明の実施例の工程フローチャート、第３図は従来法の塩基配列決定プロセスにおけるＤＮＡ断片スペクトルの変化を示す測定図、第４図は第３図の傾向を反応率として整理したグラフ、第５図，第６図はそれぞれ本発明の実施例および従来例のＤＮＡ断片スペクトルの測定図である。

代理人　弁理士　小川勝男

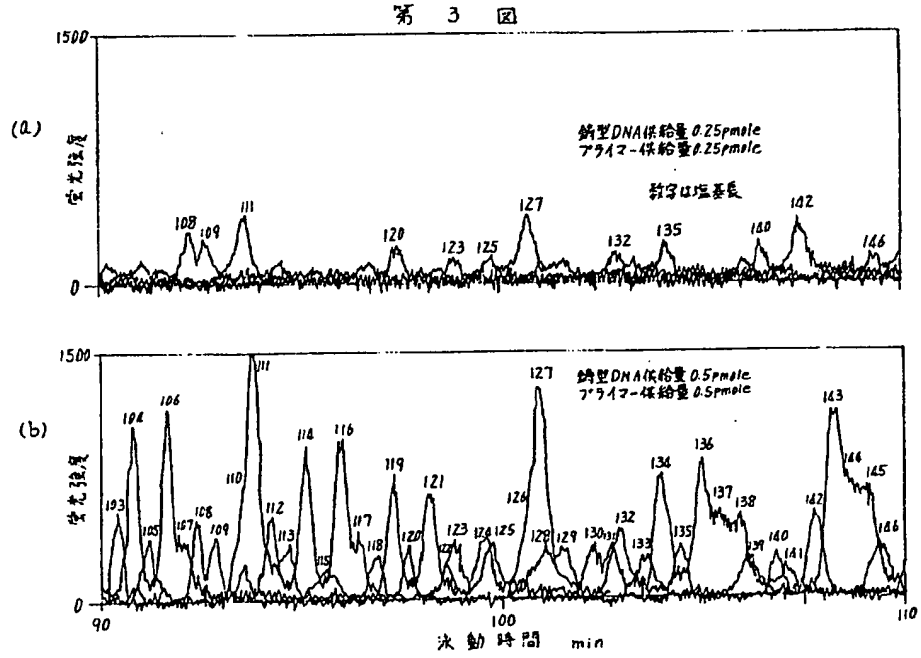
第 1 図


S
分注 1
他種DNA注入 2
インキュベーション 3
冷却 4
DNAポリメラーゼ注入 5
反応液等分 6
停止剤注入 7
インキュベーション 8
チェイス混液注入 9
インキュベーション 10
停止剤注入 11
E

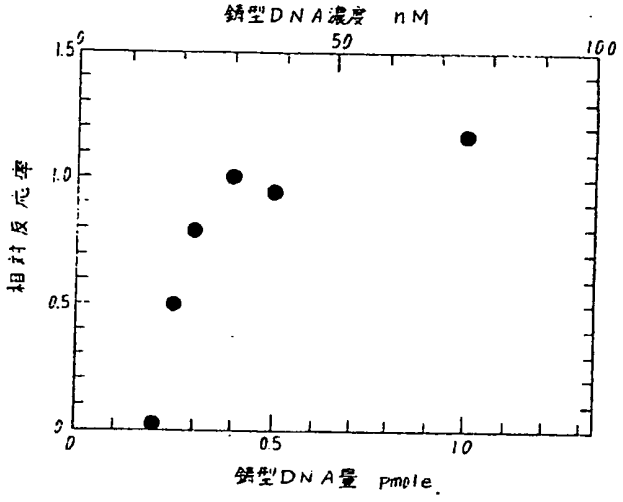
第 2 図


S
分注 1
非イオン界面活性剤注入 12
インキュベーション 3
冷却 4
DNAポリメラーゼ注入 5
反応液等分 6
停止剤注入 7
インキュベーション 8
チェイス混液注入 9
インキュベーション 10
停止剤注入 11
E

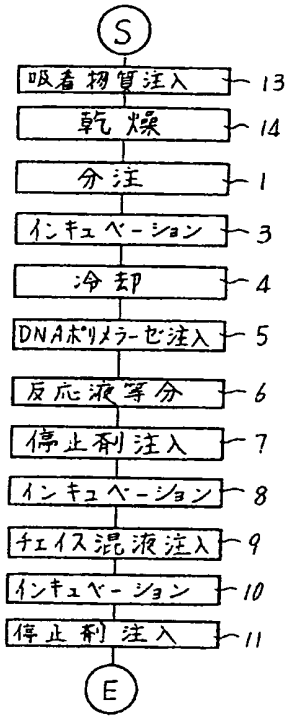
第 3 図


(a)
(b)
蛍光強度
1500
0
鋳型DNA供給量 0.25pmole
プライマー供給量 0.25pmole
数字は塩基長
鋳型DNA供給量 0.5pmole
プライマー供給量 0.5pmole
90
100
110
泳動時間 min

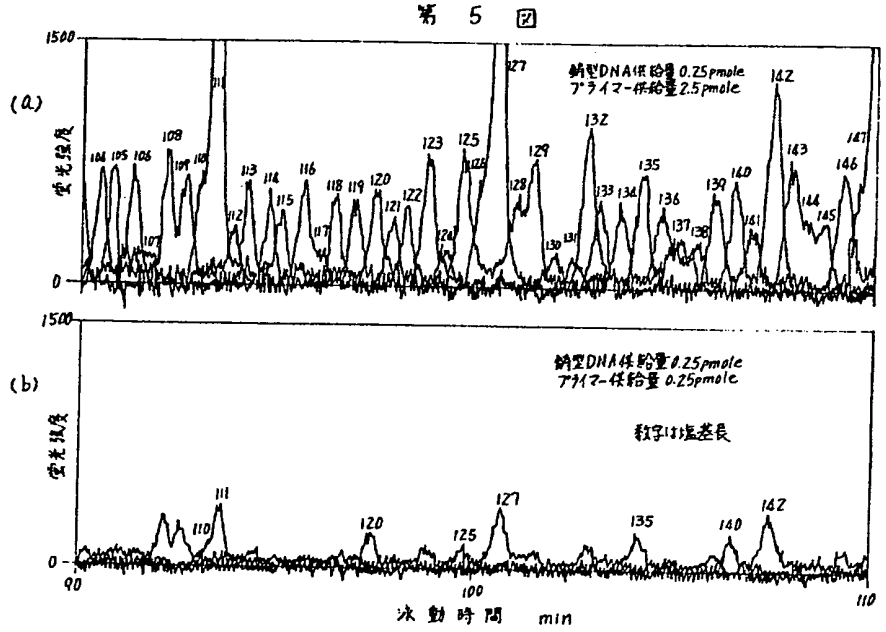
第 4 図


鋳型DNA濃度 nM
50
100
1.5
1.0
0.5
0
0
0.5
1.0
相対反応率
鋳型DNA量 Pmole

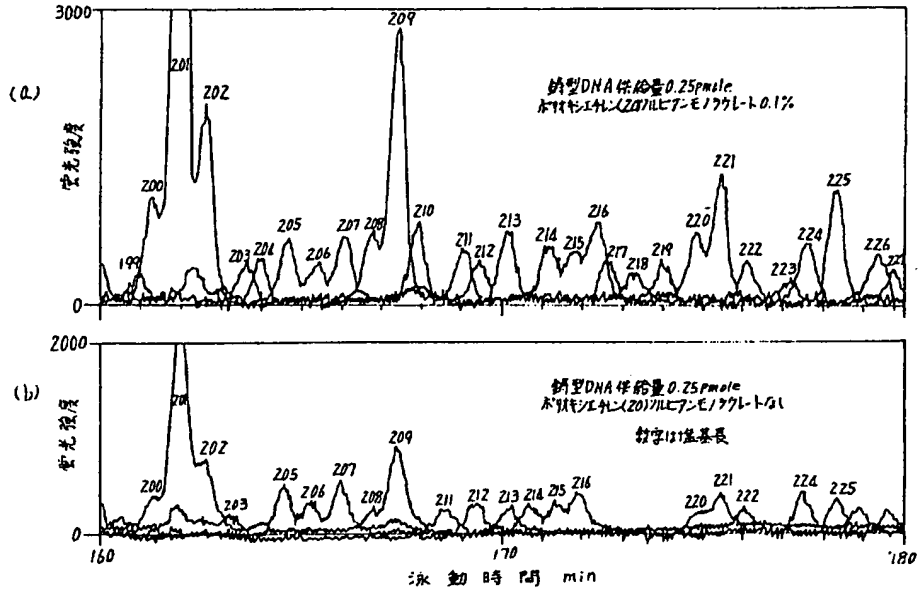
第 7 図


S
吸着担体注入
13
乾燥
14
分注
1
インキュベーション
3
冷却
4
DNAポリメラーゼ注入
5
反応液等分
6
停止剤注入
7
インキュベーション
8
チェイス混液注入
9
インキュベーション
10
停止剤注入
11
E

第 5 図


(a)
1500
0
蛍光強度
鋳型DNA供給量 0.25pmole
プライマー供給量 2.5pmole
(b)
1500
0
蛍光強度
鋳型DNA供給量 0.25pmole
プライマー供給量 0.25pmole
数字は塩基長
90
100
110
泳動時間 min

第 6 図


(a)
3000
0
蛍光強度
鋳型DNA仕込量0.25pmole
ポリオキシエチレン(20)ラウリルアンモノラウレート0.1%
(b)
2000
0
蛍光強度
鋳型DNA仕込量0.25pmole
ポリオキシエチレン(20)ラウリルアンモノラウレートなし
数字は塩基長
160
170
180
泳動時間 min